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LRP-MEDIATED MODULATION OF NEURONAL CALCIUM INFLUX VIA NMDA RECEPTORS, AND USES THEREOF

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Abstract of WO0207755

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(54) Title: LRP-MEDIATED MODULATION OF NEURONAL CALCIUM INFLUX VIA NMDA RECEPTORS, AND USES THEREOF

(57) Abstract: The present invention relates to methods of treating neurological disorders with agents that bind to low-density lipoprotein receptor-related protein (LRP) receptors. The present invention also relates to methods of modulating calcium influx and inhibiting cell death in neuronal cells by treating the neuronal cells with agents that bind to LRP. The present invention also relates to methods of identifying agents that modulate calcium influx in neuronal cells by binding to low-density lipoprotein receptor-related protein.

LRP-mediated Neuronal Calcium Modulators via NMDA Receptors, and Uses Thereof

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Field of the Invention

The present invention relates to methods of treating neurological disorders with agents that bind to low-density lipoprotein receptor-related protein (LRP) receptors. The present invention also relates to methods of modulating calcium influx and inhibiting cell death in neuronal cells by treating the neuronal cells with agents that bind to LRP. The present invention also relates to methods of identifying agents that modulate calcium influx in neuronal cells by binding to LRP.

Background of the Invention

The low-density lipoprotein receptor is one of the best studied examples of an endocytic receptor, delivering cholesterol containing lipoproteins and other ligands to acidic compartments within cells for further metabolism. A family of homologous receptors plays similar roles in various tissues, including the very low-density lipoprotein receptor (VLDL-r), the apolipoprotein E receptor 2 (APOER2), the low-density lipoprotein receptor related protein (LRP), and megalin (or GP330).

LRP is a widely expressed endocytic receptor which is strongly expressed in brain on neurons and reactive astrocytes (Rebeck, G.W., *et al., Neuron 11*:575-80 (1993)). LRP is a >600 kDa (4454 amino acid) protein cleaved in the trans-

Golgi network to form a heterodimer with a single transmembrane spanning domain, a ~515 kDa extracellular region containing 4 ligand binding repeat regions, multiple EGF and growth factor repeats, and a smaller intracellular domain containing two NPXY sequences which direct endocytosis of the receptor to clathrin coated pits (Herz, J., *et al., EMBO J. 7*:4119-27 (1988); Strickland, D.K., *et al., J. Biol. Chem. 265*:17401-4 (1990); Willnow, T.E., *et al., EMBO J. 15*:2632-9 (1996)). LRP has more than 15 identified ligands, which fall into several broad categories such as proteases, protease inhibitors, such as activated alpha-2-macroglobulin (a2M*), protease/protease inhibitor complexes, protein-lipid complexes, and other proteins and molecules such as lactoferrin. A list of the known ligands of LRP can be found in Hussain, M.M., *et al. Annu. Rev. Nutr. 19: 141-172* (1999). The 39 kDa receptor associated protein (RAP) is an endoplasmic chaperone protein tightly bound to LRP, which, when used pharmacologically, specifically blocks and prevents uptake of all known LRP ligands (Strickland, D.K., *et al., J. Biol. Chem. 265*:17401-4 (1990); Williams, S.E., *et al., J. Biol. Chem. 267*:9035-40 (1992); Medved, L.V., *et al., J. Biol. Chem. 274*:717-27 (1999)). Like other members of the LDL receptor family, LRP binds and imports these ligands into intracellular vesicles, acidified compartments where the ligand is released, and the receptor is recycled to the surface. LRP (Strickland, D.K., *et al., J. Biol. Chem. 265*:17401-4 (1990)) and APOER2 (Stockinger, W., *et al., J. Biol. Chem. 273*:32213-21 (1998)) are the only known brain receptors for a2M*, mediating clearance of protease/protease inhibitor complexes.

Summary of the Invention

The present invention is directed to a method of treating a subject in need of treatment of a neurological disorder, the method comprising: administering to the subject a pharmaceutically effective amount of an agent that binds to low-

density lipoprotein receptor-related protein (LRP) receptor of LRP on neuronal cells, and modulates calcium influx in said neuronal cells.

Additionally, the present invention is directed to a method of inhibiting cell death in neuronal cells, the method comprising: providing neuronal cells with an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor on the neuronal cells, and modulates calcium influx in the neuronal cells.

Similarly, the present invention also is directed to a method of modulating calcium influx in neuronal cells, the method comprising: treating the neuronal cells with an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor on the neuronal cells, and modulates calcium influx in the neuronal cells.

Furthermore, the present invention is directed to a method of identifying an agent that modulates calcium influx in neuronal cells by binding to low-density lipoprotein receptor-related protein (LRP) receptors on the neuronal cells, the method comprising: (a) treating neuronal cells with an agent and assaying for calcium influx; (b) treating neuronal cells with a known modulator of LRP-mediated calcium influx and the agent in (a) and assaying for calcium influx; and (c) comparing the levels of calcium influx in (a) and (b) to determine if the agent in (a) modulates calcium influx by interacting with an LRP receptor.

Details of the present invention will be clear from the description that follows.

Brief Description of the Figures

Figure 1 Shows that a2M* increases $[Ca^{2+}]_i$ specifically in neurons. Primary cultures of mouse cortex were loaded for 30 min with 1 μM indo-1/AM and imaged using a Biorad Multiphoton confocal microscope. The traces represent a time-course of intracellular calcium concentration in a field of cells in a single, representative experiment. Each trace is the average of 6 cells within the field, ± std deviation. Not all cells in the mixed cultures responded to a2M* treatment.

The cells that did respond resembled neurons morphologically and also responded to NMDA application. Non-responders had the generally flat appearance of glia and/or fibroblasts and did not respond to NMDA addition.

Figure 2 Shows that all cells in the mixed cultures expressed LRP. Immunohistochemistry using the anti-LRP antibody R777 revealed that all cells in the cultures expressed LRP (top left panel). The top right panel is a dual label with an antibody to MAP2, which was expressed exclusively in neurons (Izant, J.G. and McIntosh, J.R., *Proc. Nat. Acad. Sci. U.S.A. 77*:4741-5 (1980)). The scale bar is 25 μm.

Figure 3 Shows that the calcium increase requires extracellular calcium. Cells were placed in nominally calcium-free buffer (not containing EGTA), and a2M* was added at approximately t=150 sec. No intracellular calcium increase was observed. In fact, a small decrease was indicated. The calcium-free buffer was washed and replaced with calcium containing buffer (2 mM) at t=500 sec. After replacement of calcium, $[Ca^{2+}]_i$ levels increased to about 400 nM, similar to levels normally observed after stimulation by a2M* in calcium containing buffers. This suggests that a2M* was able to bind to LRP in the absence of calcium and initiate a calcium signaling event. The response required extracellular calcium.

Figure 4 Shows that calcium entry occurs through NMDAR channels. In this experiment, the cells were pretreated with 5 μM MK-801 for 5 minutes, and the NMDA antagonist remained in the bath throughout the procedure. At t=350 sec, a2M* (35 nM) was added to the bath, resulting in a small but insignificant increase in $[Ca^{2+}]_i$ in this field of cells. At t=700 sec, NMDA (100 μM) was added, and no change in $[Ca^{2+}]_i$ was observed. Glutamate (10 μM), however, was capable of eliciting a calcium response at t=900 sec. This is a representative trace of n=4 experiments. The trace is the mean ± std. dev. of n=15 neurons in the field.

Figure 5 Shows that a2M* increases calcium in neurons in which LRP receptors are blocked by RAP.

Figure 5A. a2M* (35 nM) was added to the bath at around 200 sec, which elicited a rapid, sustained increase in calcium. At t=650 sec, NMDA (100 μM) was added to the bath, resulting in an additional rise in calcium. The trace is the average response from 9 cell bodies in the field ± std dev.

Figure 5B. RAP (500 nM) was added to the bath at t=200 sec. RAP had no discernible effect on intracellular calcium. At t=650 sec, a2M* (35 nM) was added to the bath, but calcium was unaffected. NMDA was able to elicit a normal response in the 7 cells in this field.

Figure 6 Shows that an antibody to the ligand binding domain of LRP increases $[Ca^{2+}]_I$, but an antibody to an intracellular domain of LRP does not. This figure illustrates two experiments utilizing rabbit polyclonal antibodies directed against LRP. In the top trace (circles) R777, which recognizes the ligand binding domain of LRP, was added. Addition of R777 increased $[Ca^{2+}]_I$ in a neuron specific manner. In the bottom trace (squares), addition of R704, which recognizes an intracellular domain of LRP, was unable to elicit an increase in $[Ca^{2+}]_I$. However, subsequent addition of a2M* was able to generate a calcium response in these cells.

Detailed Description of the Preferred Embodiments

The LRP receptor is a widely expressed endocytic receptor which is strongly expressed on neuronal cells, including neurons and glial cells of the central and peripheral nervous systems (Rebeck, G.W., *et al., Neuron 11*:575-80 (1993)). A novel neuron-specific signaling role for LRP is reported herein. Namely, LRP ligand binding and receptor dimerization led to calcium influx in neuronal cells via NMDAR channels. A robust, spatially and temporally discrete calcium signal was observed in neurons treated with ligand competent a2M*,

which was blocked by RAP. Non-neuronal cells, which also expressed LRP, in the same cultures did not elicit a calcium response. The calcium signal was dependent on extracellular calcium and was blocked by the NMDA receptor antagonist MK-801 (Tolar, M., *et al., J. Neurosci. 19*:7100-10 (1999)). Calcium influx in neurons also occurred after treatment with R777, an antibody directed against the extracellular domain of LRP, and this response was also blocked with MK-801. Calcium entry did not occur after treatment with Fab fragments of R777, suggesting that receptor dimerization may be critical. These results demonstrate a novel signaling role for the multifunctional receptor LRP in neurons.

As used herein, an LRP receptor is a protein that is recognized in the art as such, and forms a heterodimer with a single transmembrane spanning domain, contains an extracellular region containing 4 ligand binding repeat regions, contains multiple EGF and growth factor repeats, and contains a smaller intracellular domain containing two NPXY sequences (where N symbolizes the amino acid asparagine, P symbolizes proline, X is any amino acid and Y symbolizes tyrosine) which direct endocytosis of the receptor to clathrin coated pits (Herz, J., *et al., EMBO J. 7*:4119-27 (1988); Strickland, D.K., *et al., J. Biol. Chem. 265*:17401-4 (1990); Willnow, T.E., *et al., EMBO J. 15*:2632-9 (1996)).

The present invention is directed to a method of treating a subject in need of treatment of a neurological disorder, the method comprising: administering to the subject a pharmaceutically effective amount of an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor on neuronal cells, and modulates calcium influx in said neuronal cells.

As used herein, the term receptor is meant to include a molecule that binds to a ligand and causes a cellular or physiological response. The receptor can be cytosolic, membrane-bound, membrane-spanning, or it can be an extracellular molecule. Additionally, the receptor can be in the form of a monomer or a multimer (i.e., dimer, trimer, or higher multimer). The term multimer encompasses a homomultimer or a heteromultimer. As used herein, the term homomultimer is

used to mean a multimer molecule where all of the individual proteins or other molecules that constitute the multimer are identical. A heteromultimer, on the other hand, is used herein to mean a multimer molecule where any of the individual proteins or other molecules that constitute the multimer are not identical. In other words, a monomeric receptor causes the physiological or cellular response solely, while the multimeric receptor may require two or more proteins or other molecules, acting in concert with one another, to cause a physiological or cellular response. Examples of molecules that can act as receptors include, but are not limited to, proteins, polysaccharides, glycoproteins, proteoglycans, nucleic acids, lipids, and lipoproteins.

Examples of cellular responses that receptors initiate or propagate, which should be obvious to one skilled in the art, include, but are not limited to ion influx or efflux, initiation of second messenger pathways, synthesis of DNA, translation of mRNA, entry of the cells into the cell cycle, arrest of the cell in the cell cycle, endocytosis, release of molecules from the cell, exocytosis, and apoptosis. The cellular response on which the current invention focuses is modulating calcium influx in the affected neuronal cell.

As used herein, modulation of calcium entry into the cytoplasm of the affected cell includes such responses as increasing or decreasing the quantity of calcium ions that normally enter the cell from the extracellular environment, in conjunction with another stimulus. Likewise, modulation of calcium entry into the cytoplasm of the affected cell also includes such responses as increasing or decreasing the quantity of calcium ions that normally enter the cell from the extracellular environment, in the absence of another stimulus. Additionally, modulation of calcium influx is meant to encompass increasing or decreasing the quantity of release or uptake of calcium ions from or to intracellular stores, such as mitochondria, in conjunction with another stimulus. Likewise, modulation of calcium influx is also meant to encompass increasing or decreasing the quantity of release or uptake of calcium ions from or to intracellular stores, such as mitochondria, in the absence of another stimulus.

Preferably, upon binding of an agent with LRP, the channel through which calcium influx in the neuronal cells is mediated is not LRP. More preferably, the channel through which calcium influx in the neuronal cells is mediated is through a class of receptors that binds to the ligand N-methyl-D-aspartate (NMDA), dubbed NMDA receptors. NMDA receptors are ligand-gated ion channels that are a subclass of the larger family of glutamate receptors. A ligand-gated channel is a receptor that binds a ligand and subsequently opens to allow the flow of ions, such as Na^+, K^+ or Ca^{2+}, into or out of the cell. In addition to ligand binding, the flow of ions through ligand-gated channels is also controlled by the voltage potential across the plasma membrane separating the cytosol and the extracellular space. Specifically, the NMDA receptor is a channel for Ca^{2+}, and it is thought to be responsible for the induction of long-term potentiation (LTP) and long-term depression (LTD). LTP is the phenomenon where a postsynaptic neuron has a prolonged, increased response to a presynaptic stimulus. LTP is thought to, at least partially, account for cellular and physiological memory and learning. Conversely, LTD is the phenomenon where a postsynaptic neuron has a prolonged decrease response to a presynaptic stimulus.

One embodiment of the current invention is that the agent that binds to LRP, causes dimerization of the LRP receptors. Subsequently, this dimerization process regulates calcium influx in the neuronal cells. As used herein, dimerization is the process that is well-recognized in the art where two separate proteins form an association. The dimer formed may be a heterodimer or a homodimer. The association forming the dimer can be temporary or permanent. Furthermore, the association of the two proteins can serve to enhance or diminish the normal function or signaling capacity of each of the two proteins. Alternatively, the association of the two proteins can lead to a completely different function, e.g., second messenger propagation, than the normal function of either of the two proteins.

As used herein, the term agent, ligand or compound is intended to mean a protein, nucleic acid, carbohydrate, lipid or a small molecule. The types of

agents or compounds which can be envisioned are limited only by their ability to bind to LRP and mediate calcium influx, particularly through the NMDA receptor channels. LRP has at least four documented binding sites. Each of these four binding sites has its own ligand binding specificity domains, such that the various ligands that LRP binds do not bind to the same binding domain. For example, although lactoferrin is a ligand of LRP, there are binding sites on LRP that are unresponsive to lactoferrin. Thus, in one embodiment, the agent binds to a site on LRP that is unresponsive to lactoferrin. In another embodiment, the agent can bind to a site on the LRP that does not bind to amyloid precursor protein (APP). Similarly, the agent that binds to LRP and modulates calcium does not interfere with the amount or rate of binding of APP to LRP. Alternatively, the agent binds to a site on LRP that is unresponsive to lactoferrin, and the agent also does not bind to the APP binding site. Preferably, agents of the present invention include agents selected from, but are not limited to, protein-lipid complexes, proteases, protease inhibitors, protease-inhibitor complexes, intracellular proteins, small molecules, LRP receptor antibodies, and LRP-interacting proteins. Examples of members of the aforementioned classes, which should be obvious to one skilled in the art include, but are not limited to, protein-lipid complexes involved in lipid and/or cholesterol metabolism such as apolipoprotein, proteases such as plasminogen, protease inhibitors such as activated alpha-2-macroglobulin, proteins such as beta-amyloid precursor protein, small molecules such as aminoglycosides (e.g., gentamicin), LRP receptor antibodies. and receptor associated protein (RAP) or compounds based on the structure of RAP. Accordingly, preferable agents of the present invention include, but are not limited to, activated alpha-2-macroglobulin, apolipoprotein E, and apolipoprotein E4. A preferred agent of the current invention is activated alpha-2-macroglobulin.

The current invention can be useful in treating a subject in need of treatment of a neurological disorder where aberrant calcium influx in neuronal cells is either causal or symptomatic. A neurological disorder, as used in the current context, should be obvious to one skilled in the art, but is meant to include

any abnormal physical or mental behavior or experience where neuronal cells are involved in the etiology of the disorder, or are affected by the disorder. As used herein, neurological disorders encompass disorders affecting the central and peripheral nervous systems, and include such afflictions as memory loss, stroke, dementia, personality disorders, gradual, permanent or episodic loss of muscle control. Examples of neurological disorders for which the current invention can be used include, but are not limited to: Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, amyotrophic lateral sclerosis, epilepsy, and stroke. More preferably, the current invention can be used to treat Alzheimer's Disease.

As used herein, the term subject can be used to mean an animal, preferably a mammal including a human or non-human. The term patient is used to indicate a subject in need of treatment of a neurological disorder.

The treatment envisioned by the current invention can be used for patients with a pre-existing neurological condition, or for patients pre-disposed to a neurological disorder. Additionally, the method of the current invention can be used to correct cellular or physiological abnormalities involved with a neurological disorder in patients, and/or to alleviate symptoms of a neurological disorder in patients, or as a preventative measure in patients.

The present invention is further directed to a method of inhibiting cell death in neuronal cells, the method comprising: providing neuronal cells with an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor and modulates calcium influx in the neuronal cells. The invention can be practiced *in vitro* or *in vivo*. As used herein, cell death includes a process or event that causes the cell to cease or diminish normal metabolism *in vivo* or *in vitro*. The various forms and signs of cell death are obvious to those skilled in the art, but examples of cell death include, but are not limited to, programmed cell death (i.e., apoptosis), gradual death of the cells as occurs in diseased states (i.e., necrosis), and more immediate cell death such as acute toxicity. The inhibition of cell death for which the current invention provides can be a complete or partial inhibition of cell death. Likewise, the inhibition of cell death for which the current invention

provides can be a complete or partial reversal of the process of cell death. Preferably, the present invention inhibits cell death by modulating calcium influx in neuronal cells. As the current invention contemplates, modulation of calcium influx has been previously described herein.

The present invention provides for inhibiting cell death by modulating calcium influx in neuronal cells. Preferably, the channel through which calcium influx in the neuronal cells is mediated is not LRP. More preferably, the channel through which calcium influx in the neuronal cells is mediated is through the NMDA class of receptors.

Agents of the current invention useful for inhibiting neuronal cell death include any agent that binds to LRP and subsequently modulates calcium influx in neuronal cells. Preferably, the agents of the current invention useful for inhibiting cell death include, but are not limited to, receptor associated protein (RAP), small molecules or peptides that mimic RAP, LRP receptor antibodies, and proteins that interact with LRP. Such agents have been previously described herein.

The present invention also is directed to a method of modulating calcium influx in neuronal cells, the method comprising: treating the neuronal cells with an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor on neuronal cells and modulates calcium influx in the neuronal cells. Preferably, the channel through which calcium influx in the neuronal cells is mediated is not LRP. More preferably, the channel through which calcium influx in the neuronal cells is mediated is through the NMDA class of receptors. As the current invention contemplates, modulation of calcium influx has previously been described. The invention can be practiced *in vitro* or *in vivo*.

Preferably, agents that interact with LRP on neuronal cells and modulate calcium influx include, but are not limited to protein-lipid complexes, proteases, protease inhibitors, protease-inhibitor complexes, proteins, small molecules, LRP receptor antibodies, and proteins that interact with LRP. Such agents have been previously described herein. Furthermore, the types of agents that can be envisioned include agonists and antagonists of LRP and are limited only by their

ability to bind to LRP and modulate calcium influx, particularly through the NMDA receptor channels. As used herein, an agonist is a protein, nucleic acid, carbohydrate, lipid or a small molecule that binds to LRP and mimics the calcium influx that activated alpha-2-macroglobulin elicits under similar, or identical, conditions. The cellular response that the agonist mimics does not have to be identical in magnitude, duration or character. As used herein, an antagonist is a protein, nucleic acid, carbohydrate, lipid or a small molecule that binds to LRP and attenuates, or reverses the calcium influx that activated alpha-2-macroglobulin elicits under similar, or identical, conditions. The cellular response that the antagonist prevents does not have to be a total prevention or reversal of the response that the ligand elicits.

Agents of the present invention that increase calcium influx in neuronal cells are agonists or stimulators of LRP. As used herein, the term stimulator is meant to include any agent that produces an increase in calcium movement into the cell. Thus, a stimulator of LRP-mediated calcium movement is any agent that binds to LRP and causes an increase in calcium movement into or out of the cell. A preferred agent of the current invention that is an agonist of LRP and modulates calcium influx in neuronal cells is activated alpha-2-macroglobulin. Similarly, preferable agonists of LRP that are used to modulate calcium influx in neuronal cells also include antibodies that increase LRP-mediated calcium influx in neurons through the NMDA receptor channels, such as R777. The antibody R777 is an antibody that binds to LRP and can be used to block the binding of activated alpha-2-macroglobulin and other ligands or agents that bind to LRP. The R777 antibody was obtained from the fusion of spleen cells of mice, which had been immunized with activated a2M, with the myeloma cell line P3-X63-Ag8.653. The production and binding specificity of R777 towards LRP has been previously described in Strickland, D.K., *et al., Biochemistry* 27: 1458-1466 (1988), and Strickland, D.K., *et al., J. Biol. Chem.* 265:17401-17404 (1990).

Agents of the present invention that decrease calcium influx in neuronal cells are antagonists or inhibitors of LRP. As used herein, the term inhibitor is

meant to include any agent that produces a complete or partial blocking of calcium movement into the cell. Thus, an inhibitor of LRP-mediated calcium movement is any agent that binds to LRP and produces a complete or partial blocking of calcium movement into the cell. Most preferably the agents of the current invention that are antagonists of LRP modulate calcium influx in neuronal cells are receptor associated protein (RAP), antibodies that inhibit LRP-mediated calcium influx in neurons through the NMDA receptor channels, and small molecules or peptides that mimic RAP.

The antibodies used in the invention can be, but are not limited to, chimeric, humanized, and human and nonhuman monoclonal and polyclonal antibodies. Antibodies may be used as an isolated whole antibody, or can be used as a source for generating antibody fragments which contain the antigen binding site of the antibody. Examples of such antibody fragments include, but are not limited to the F_v, the F(ab), the $F(ab)_2$, fragment and single chain antibodies. Various methods known in the art can be used to generate such whole antibodies or antibody fragments without undue experimentation. For example, a polypeptide of interest or an antigenic fragment thereof can be administered to an animal to induce the production of sera containing polyclonal antibodies. Monoclonal antibodies can be prepared using a wide of techniques known in the art including the use of hybridoma and recombinant technology. *See, e.g.*, Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). Recombinant techniques are preferred for generating large quantities of antibodies, antibody fragments and single chain antibodies, as described, for example, in Pluckthum, *Bio/Technology 10*:163-167 (1992); Carter *et al., Bio/Technology 10*:167-170 (1992); and Mullinax *et al., Biotechniques 12*:864-869 (1992). In addition, recombinant techniques may be used to generate heterobifunctional antibodies.

Furthermore, the present invention is directed to a method of identifying an agent that modulates calcium influx in neuronal cells by binding to low-density lipoprotein receptor-related protein (LRP) receptor on the neuronal cells, the method comprising: (a) treating neuronal cells with an agent and assaying for calcium influx; (b) treating neuronal cells with a known modulator of LRP-mediated calcium influx and the agent in (a) and assaying for calcium influx; and (c) comparing the levels of calcium influx in (a) and (b) to determine if the agent in (a) modulates calcium influx by interacting with an LRP receptor. Preferably, the channel through which calcium influx in the neuronal cells is mediated is not LRP. More preferably, the channel through which calcium influx in the neuronal cells is mediated is through the NMDA class of receptors. Accordingly, in one preferred embodiment of the current invention, the known modulator of LRP-mediated calcium influx is an NMDA receptor channel antagonist. Examples of NMDA receptor channel antagonists include, but are not limited to, MK-801, D(-)-2- Amino- 5- phosphonopentanoic acid, D(-)- 2- Amino- 4-phosphonobutyric acid, ketamine, ifenprodil or phencyclidine. More preferably, the NMDA receptor channel antagonist is MK-801. The pharmacological agent MK-801 is also known as dizocilpine and is described in Woodruff G.N. *et al.*, Neuropharmacology 26: 903-9 (1987). As used herein, an NMDA receptor channel antagonist is a protein, nucleic acid, carbohydrate, lipid or a small molecule that binds to an NMDA receptor and blocks, attenuates, or reverses the calcium influx that N-methyl-D-aspartate elicits under similar, or identical, conditions.

In one embodiment of the current invention, the method of identifying an agent that modulates calcium influx in neuronal cells is performed on a single population of cells, and (b) is performed on the identical population after the agent in (a) is removed. In another embodiment of the invention, the method of identifying an agent that modulates calcium influx in cells is performed on two nearly identical populations of cells, under the same culture conditions, where (a) is performed on one population and (b) is performed on another population, and

(c) is a comparison of the levels of calcium influx between the two populations of cells.

As used herein, assaying for calcium influx can be accomplished by using any means that can detect differences in intracellular or extracellular calcium levels. Such means, which should be obvious to one of ordinary skill on the art, include, but are not limited to, the use of fluorescent dyes in conjunction with microscopy (calcium imaging) (Grynkiewicz, G., *et al., J. Biol. Chem. 260*:3440-50 (1985)), enzyme-linked immunosorbent assays (ELISA), radioactively-labeled isotopes, and detecting local or systemic changes in membrane potential or voltage.

The types of agents that can be tested can be proteins, nucleic acids, carbohydrates, lipids or small molecules. The types of agents or compounds which can be envisioned are limited only by their ability to bind to LRP and modulate LRP-mediated calcium influx in neurons through the NMDA receptor channels.

The agents of the present invention may be identified and/or prepared according to any of the methods and techniques known to those skilled in the art. These agents, particularly peptide agents and antibody agents, may occur or be produced as monomer, dimers, trimers, tetrameres or multimers. Such multimers can be prepared using enzymatic or chemical treatment of the native receptor molecules or be prepared using recombinant techniques. Preferably, the agents of the present invention are selected and screened at random or rationally selected or designed using protein modeling techniques.

For random screening, candidate agents are selected at random and assayed for their ability to bind to LRP and cause calcium influx in neurons. Any of the suitable methods and techniques known to those skilled in the art may be employed to assay candidate agents.

For rational selection or design, the agent is selected based on the configuration of the LRP binding site found on an LRP ligand, e.g. a2M*, or a ligand binding site found on the LRP. Any of the suitable methods and techniques

known to those skilled in the art may be employed for rational selection or design. For example, one skilled in the art can readily adapt currently available procedures to generate antibodies, peptides, pharmaceutical agents and the like capable of binding to a specific peptide sequence of LRP. Illustrative examples of such available procedures are described, for example, in Hurby *et al.*, "Application of Synthetic Peptides: Antisense Peptides," in *Synthetic Peptides, A User's Guide*, W.H. Freeman, NY, pp. 289-307 (1992); Kaspczak *et al., Biochemistry 28*:9230 (1989); and Harlow, *Antibodies*, Cold Spring Harbor Press, NY (1990).

The agents of the present invention can alternatively be identified using modification of methods known in the art. For example, suitable peptide agents may be identified using the filter binding assay described by Mischak *et al.* (Mischak *et al., J. Gen. Virol. 69*:2653-2656 (1988) and Mischak *et al., Virology 163*:19-25 (1988)), wherein the peptide is applied to a suitable membrane, such as nitrocellulose, and the membrane is saturated with a detergent mixture in order to block any non-specific binding. The treated membrane is then incubated with labeled LRP (labeled with ^{125}I-iodine), to check the specific binding. After washing and drying of the membrane, specific binding can be visualized by autoradiography.

Formulations and Methods of Administration

As used herein, "a pharmaceutically effective amount" is intended an amount effective to elicit a cellular response that is clinically significant, without excessive levels of side effects.

A pharmaceutical composition of the invention is thus provided comprising an agent of the invention useful for treatment of a neurological disorder and a pharmaceutically acceptable carrier or excipient.

It will be desirable or necessary to introduce the pharmaceutical compositions directly or indirectly to the brain. Direct techniques usually involve placement of a drug delivery catheter into the host's ventricular system to bypass

the blood-brain barrier. Indirect techniques, which are generally preferred, involve formulating the compositions to provide for drug latentiation by the conversion of hydrophilic drugs into lipid-soluble drugs. Latentiation is generally achieved through blocking of the hydroxyl, carboxyl, and primary amine groups present on the drug to render the drug more lipid-soluble and amenable to transportation across the blood-brain barrier. Alternatively, the delivery of hydrophilic drugs can be enhanced by intra-arterial infusion of hypertonic solutions which can transiently open the blood-brain barrier.

The blood-brain barrier (BBB) is a single layer of brain capillary endothelial cells that are bound together by tight junctions. The BBB excludes entry of many blood-borne molecules. In the invention, the agent can be modified for improved penetration of the blood-brain barrier using methods known in the art. Alternatively, a compound with increase permeability of the BBB can be administered to the subject. RMP-7, a synthetic peptidergic bradykinin agonist was reported to increase the permeability of the blood-brain barrier by opening the tight junctions between the endothelial cells of brain capillaries (Elliott, P.J. *et al., Exptl. Neurol. 141*:214-224 (1996)).

The invention further contemplates the use of prodrugs which are converted in vivo to the therapeutic compounds of the invention (Silverman, R.B., "The Organic Chemistry of Drug Design and Drug Action," Academic Press, Ch. 8 (1992)). Such prodrugs can be used to alter the biodistribution (e.g., to allow compounds which would not typically cross the blood-brain barrier to cross the blood-brain barrier) or the pharmacokinetics of the therapeutic compound. For example, an anionic group, e.g., a sulfate or sulfonate, can be esterified, e.g, with a methyl group or a phenyl group, to yield a sulfate or sulfonate ester. When the sulfate or sulfonate ester is administered to a subject, the ester is cleaved, enzymatically or non-enzymatically, to reveal the anionic group. Such an ester can be cyclic, e.g., a cyclic sulfate or sultone, or two or more anionic moieties can be esterified through a linking group. An anionic group can be esterified with moieties (e.g., acyloxymethyl esters) which are cleaved to reveal an intermediate

compound which subsequently decomposes to yield the active compound. Furthermore, an anionic moiety can be esterified to a group which is actively transported in vivo, or which is selectively taken up by target organs. The ester can be selected to allow specific targeting of the therapeutic moieties to particular organs, as described below for carrier moieties.

The therapeutic compounds or agents of the invention can be formulated to cross the blood-brain-barrier, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Pat. Nos. 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs thus providing targeted drug delivery (Ranade, J., *Clin. Pharmacol. 29*:685 (1989)). Exemplary targeting moieties include folate or biotin (U.S. Pat. No. 5,416,016), mannosides (Umezawa *et al., Biochem. Biophys. Res. Comm. 153*:1038 (1988)), antibodies (Bloeman *et al., FEBS Lett.* 357:140 (1995); Owais *et al., Antimicrob. Agents Chemother. 39*:180 (1995)), surfactant protein A receptor (Briscoe *et al., Am. J. Physiol. 1233*:134 (1995)), gp 120 (Schreier *et al., J. Biol. Chem. 269*:9090 (1994); Killion and Fidler, *Immunomethods 4*:273 (1994)).

The pharmaceutical composition can be administered orally, nasally, parenterally, intrasystemically, intraperitoneally, topically (as by drops or transdermal patch), bucally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is intended, but not limited to, a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

A pharmaceutical composition of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or

vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

The compositions of the present invention can also contain adjuvants such as, but not limited to, preservatives, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms can be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It can also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of the drugs, it is desirable to slow the absorption from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, can depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Solid dosage forms for oral administration include, but are not limited to, capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compounds are mixed with at least one item pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, acetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and I) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form can also comprise buffering agents.

Solid compositions of a similar type can also be employed as fillers in soft and hardfilled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They can optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms can contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Suspensions, in addition to the active compounds, can contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth, and mixtures thereof.

Topical administration includes administration to the skin or mucosa, including surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, can be prepared as a dry powder which can be pressurized or non-pressurized. In nonpressurized powder compositions, the active ingredients in finely divided form can be used in admixture with a larger-sized pharmaceutically acceptable inert carrier comprising particles having a size, for example, of up to 100 μm in diameter. Suitable inert carriers include sugars such as lactose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10 μm.

Alternatively, the composition can be pressurized and contain a compressed gas, such as nitrogen or a liquefied gas propellant. The liquefied propellant medium and indeed the total composition is preferably such that the

active ingredients do not dissolve therein to any substantial extent. The pressurized composition can also contain a surface active agent. The surface active agent can be a liquid or solid non-ionic surface active agent or can be a solid anionic surface active agent. It is preferred to use the solid anionic surface active agent in the form of a sodium salt.

The compositions of the present invention can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to the compounds of the invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art (*see*, for example, Prescott, Ed., *Meth. Cell Biol. 14*:33 *et seq* (1976)).

Dosaging

One of ordinary skill will appreciate that effective amounts of the agents of the invention can be determined empirically and can be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt, ester or prodrug form. The agents can be administered to a subject, in need of treatment of a neurological disorder, as pharmaceutical compositions in combination with one or more pharmaceutically acceptable excipients. It will be understood that, when administered to a human patient, the total daily usage of the agents or composition of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors: the type and degree of the cellular response to be achieved; activity of the specific agent or

composition employed; the specific agents or composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the agent; the duration of the treatment; drugs used in combination or coincidental with the specific agent; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the agents at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosages until the desired effect is achieved.

For example, satisfactory results are obtained by oral administration of the compounds at dosages on the order of from 0.05 to 10 mg/kg/day, preferably 0.1 to 7.5 mg/kg/day, more preferably 0.1 to 2 mg/kg/day, administered once or, in divided doses, 2 to 4 times per day. On administration parenterally, for example by i.v. drip or infusion, dosages on the order of from 0.01 to 5 mg/kg/day, preferably 0.05 to 1.0 mg/kg/day and more preferably 0.1 to 1.0 mg/kg/day can be used. Suitable daily dosages for patients are thus on the order of from 2.5 to 500 mg p.o., preferably 5 to 250 mg p.o., more preferably 5 to 100 mg p.o., or on the order of from 0.5 to 250 mg i.v., preferably 2.5 to 125 mg i.v. and more preferably 2.5 to 50 mg i.v.

Dosaging can also be arranged in a patient specific manner to provide a predetermined concentration of the agents in the blood, as determined by techniques accepted and routine in the art (HPLC is preferred). Thus patient dosaging can be adjusted to achieve regular on-going blood levels, as measured by HPLC, on the order of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein can be made without departing from the scope of the invention or any embodiment thereof.

The following Example serves only to illustrate the invention, and is not to be construed as in any way to limit the invention.

Example

Materials and Methods

Primary cultures of mouse cortex were prepared from embryonic day 15-17 CD1 mice. The cortices were isolated and triturated in Ca^{2+} free PBS and plated onto 35 mm poly-lysine coated culture dishes at a density of $2x10^6$ cells/ml in neurobasal medium containing 10% fetal bovine serum (Intergen, NY), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptamycin. After 45-60 min at 37°C, supernatants containing unattached cells were removed, and attached cells were incubated in neurobasal medium supplemented with 1X B27 (Gibco, Gaithersburg, MD). After 48 hrs, 5 μg/ml cytosine-β-D-arabinofuranoside (Sigma, St. Louis) was added for 48 hrs in serum containing media, and then the media was replaced with neurobasal plus B27. Cultures were used between 7-14 days after plating. Although highly enriched for neurons, the cultures contained some non-neuronal cells.

Alpha-2-macroglobulin (Sigma) was activated (a2M*) by incubating with 100 mM methylamine at pH=7.6 in PBS for 1 hr at room temperature, followed by dialysis in PBS for 24 hours at 4°C, with at least 3 buffer changes. Native a2M was treated identically except for the addition of methylamine.

For calcium imaging experiments, indo-1/AM (Calbiochem, La Jolla, CA) was mixed with 20% pluronic F- 127 (Molecular Probes, Eugene, Oregon) in DMSO and then added to the culture dishes at a final concentration of 1 μM indo-1/AM and 0.02% pluronic F-127 for 30 min (Grynkiewicz, G., *et al., J. Biol. Chem. 260*:3440-50 (1985)). The cells were then washed and maintained in Hanks Balanced Salt Solution (HBSS), supplemented with 1 g/L glucose, pH=7.4.

The dishes were placed on the stage of an upright microscope (Olympus BX50WI), and imaged with a multi-photon confocal microscope (Biorad, Hercules, CA). A femtosecond pulsed Ti:Saphire laser (Spectra Physics, Mountain View, CA) tuned to 725 nm, provided approximately 300 mW of excitation power. External PMTs (Hamamatsu, Hamamatsu City, Japan) which

did not require de-scanning the emission signal were used to capture the two wavelength channels which were discriminated with interference filters corresponding to 390 nm, 65 nm band pass, and 495 nm, 20 nm band pass (Chroma Technology Corp., Brattleboro, Vermont). A 60x water immersion objective, NA=0.9 (Olympus, Japan), was used to view the cells.

Time course experiments were performed by acquiring an image pair (512x512 pixels, 8 bits per pixel) at a relatively slow rate (generally, 0.2 Hz) and saving the images to disk. Regions of interest (ROI) within an image were selected, corresponding to the cell bodies of single cells. The average intensity from within each ROI was obtained for each emission wavelength, the appropriate background level was subtracted, and the ratio was calculated. The ratio reflects changes in intracellular calcium ($[Ca^{2+}]_i$), independently of excitation strength, concentration of indo-1, volume of the cell, or the optical path. The ratios were converted to calcium concentration after calibrating the dye in vitro with a series of calcium buffers (Molecular Probes), and plotted as a function of time.

Fab fragments were generated from the polyclonal antibody as follows. R777 (Strickland, D.K., *et al., Biochemistry* 27: 1458-1466 (1988), and Strickland, D.K., *et al., J. Biol. Chem.* 265:17401-17404 (1990)) was dialyzed against 20 mM sodium phosphate, 10 mM EDTA, pH 7.0, and mixed with 0.5 mL Pierce immobilized papain in 20 mM sodium phosphate, 10 mM EDTA, pH 7.0 containing 20 mM cysteine. Digestion was carried out at 37°C for 12 h with gentle mixing. Following digestion, the digest was applied to Protein A Sepharose, and the nonbinding Fab fragments were collected. The Fab fragments were analyzed by immunoblotting cell extracts (using 5 μg/ml), which revealed positive reactivity against LRP and no other proteins.

Statistics were performed using a paired Student's t-test. Data from each cell within an experiment was averaged, and statistics were performed based on the number of experiments. Data are expressed as mean ± standard deviation.

Results

Primary cultures of mouse cortex were loaded with the fluorescent calcium indicator indo-1/AM. Addition of methylamine-activated a2M (a2M*, 35 nM) elicited an increase in $[Ca2+]_I$ in a subset of cells within the mixed cultures (Figure 1). a2M* increased intracellular calcium in responsive cells from 88 ± 29 nM to 396 ± 22 nM (n=24 expts, 211 cells, $p<0.001$). Unresponsive cells did not exhibit a significant increase in $[Ca2+]_I$, (106 ± 22 nM vs 107 ± 18 nM, n = 8 expts, 26 cells, NS, $p>0.05$).

Morphologically, the responding cells resembled neurons, and the non-responding cells had the appearance of glia or fibroblasts. To help distinguish the identity of cells after an experiment, NMDA (100 μM) was added to the bath. Non-neuronal cells generally do not respond to NMDA (Beaman-Hall, C.M., *et al., J. Neurochem. 71*:1993-2005 (1998)), whereas neurons that do express NMDA receptors allow calcium entry in the presence of NMDA (Grant, E.R., *et al., J. Biol. Chem.* 272:647-56 (1997)). Using this criterion, the responding cells were all identified as neurons. Greater than 95% of NMDA responsive cells responded to a2M* (203 out of 208 cells), whereas greater than 90% of all non-NMDA responsive cells failed to show a calcium response to a2M* (67 of 72). This functional marker was confirmed using immunocytochemistry for MAP-2 to identify neurons (Figure 2).

The time course and magnitude of the response to bath application of a2M* varied to some extent even among neurons within a field. The calcium response occurred within several tens of seconds after ligand addition, and, in most cases, the response was sustained for several tens of minutes until the end of the experiment. However, occasionally the calcium response was transient, returning to baseline within several minutes. No consistent difference in these subpopulations in terms of response to NMDA or in morphology was noted.

Next the specificity of the response for the activated form of a2M, a2M* was examined. Treatment of neurons with native a2M (70 nM) had no effect on

intracellular calcium (139 ± 80 nM vs 144 ± 80 nM, n= 4 expts, 54 cells, NS, p>0.05). Likewise, to test the possibility that residual methylamine was initiating the increase in calcium, methylamine was added at concentrations up to 100 μM directly to the cultures with no effect on intracellular calcium. Thus, activated a2M appears to be critical for the calcium response.

To test the possibility that the a2M* induced increase in intracellular calcium is an indirect effect of synaptic activity in the cultures, a2M* was added in the presence of 2-5 μM tetrodotoxin (TTX). At this concentration, the cultured neurons are unable to generate action potentials. However, a2M* was capable of eliciting a calcium response even in the presence of TTX. This result indicates that the observed calcium response elicited by a2M* is not an indirect result of synaptic glutamate release.

Although the time-course of the calcium response in neurons was not suggestive of calcium release from intracellular stores, this hypothesis was tested by adding a2M* in the absence of extracellular calcium (Figure 3). Under this condition, a2M* (35 nM) was unable to increase intracellular calcium (82 ± 20 nM vs 64 ± 14 nM, n=3 expts, 31 cells, NS, p>0.05). This indicates that the observed calcium entry is not from the release of calcium from intracellular stores. When the calcium-free buffer was replaced with a calcium-containing buffer, intracellular calcium increased to typical stimulated levels (266 ± 70 nM, p<0.05). This suggests that 1) the a2M* was able to bind to its receptor in the absence of calcium; 2) receptor-mediated processes allowing calcium entry were activated in the absence of calcium; and 3) the source of the calcium entry is from the extracellular environment through plasma membrane calcium channels. Consistent with this idea, pretreating the cultures with the non-specific calcium channel blockers $NiCl_2$, (2-5 mM, 89 ± 8 nM vs 107 ± 23 nM, n=5 expts, 25 cells, NS, p>0.05) or $CoCl_2$ (86 ± ??nM vs 107 ± 23 nM, 5 mM, n=4 expts, 44 cells, NS, p>0.05)) abolished the calcium response to a2M*.

To test whether NMDAR channels might be involved in the response, cultures were pretreated with 5 μM MK-801, a potent NMDA receptor

antagonist. This treatment abolished the $[Ca^{2+}]_I$ response mediated by a2M* (Figure 4) (84 ± 12 nM vs 113 ± 26 nM, n=4 expts, 60 cells, NS, $p>0.05$). This result demonstrates that the calcium signal observed by activation of LRP with a2M* is mediated by calcium entry through NMDA receptors. This also explains the observation that the response is specific for neurons in the mixed cultures.

A variety of other channel antagonists were tested, however none were able to prevent the a2M*-stimulated calcium increase when used alone, including nimodipine, ω-conotoxin, and ω-agatoxin IVA, as shown below (Table 1).

Table 1

Channel Antagonist	Type of channel affected	Result
Ca^{2+} -removal	All Ca^{2+} channels	Blocked Ca^{2+} response
$NiCl_2$(2-5 mM)	All Ca^{2+} channels	Blocked Ca^{2+} response
$CoCl_2$ (5 mM)	All Ca^{2+} channels	Blocked Ca^{2+} response
MK801	NMDAR channels	Blocked Ca^{2+} response
ω-agatoxin IVA (2 μM)	P/Q-type Ca^{2+} channels	No effect
nimodipine (5 μM)	L-type Ca^{2+} channels	No effect
ω-conotoxin (1 μM)	N–type Ca^{2+} channels	No effect
tetrodotoxin (5 μM)	Na^{2+} channels	No effect

Table 1 The effect of channel blockers on the a2M*-mediated calcium response. The table lists the channel blockers used (at the indicated concentrations), as well as the target of the blockers, and the experimental result. Each experimental test was performed in at least three cultures.

a2M*-induced calcium influx was examined to test if this phenomenon was mediated by LRP. Pre-incubation with a specific physiologic inhibitor of LRP, receptor associated protein (RAP, 500 μM), blocked the response to a2M* (114 ± 17 nM vs 125 ± 9 nM, n=4 expts, 27 cells, NS, $p>0.05$, Figure 5). RAP blocks ligand-receptor interactions with all members of the LDL receptor family proteins. Next, an anti-LRP antibody was used which specifically interacts with LRP but not other members of the LDL receptor family. The rabbit polyclonal antibody R777, directed against the ligand binding repeat region of LRP, was

added to neuronal cultures at a concentration of 10 μg/ml (Figure 6). This resulted in an immediate, marked increase in neuronal calcium (84 ± 13 nM vs 728 ± 426 nM, n=8 expts, 92 cells, $p<0.01$) but no response in non-neuronal cells. The response to R777 was blocked completely when the cultures were pretreated with 5 μM MK-801 (102 ± 12 nM vs 120 ± 4 nM, n=3 expts, 61 cells, NS, $p<0.05$). The rabbit polyclonal antibody R704 (Strickland, D.K., *et al., J. Biol. Chem.* 265:17401-17404 (1990)), which is directed against a C-terminal portion of LRP, did not elevate intracellular calcium (86±5 nM vs 120±5 nM, n=3 expts, 29 cells, NS, $p>0.05$). Thus, the ability of antibody R777 to recognize the ligand binding region of LRP activated the calcium response.

Both a2M*, which is tetrameric, and the bivalent R777 antibody potentially lead to dimerization of the ligand binding domains of LRP. To test the possibility that dimerization of the receptor or cross-linking of the ligand binding sites is important for the calcium response in neurons, Fab fragments were derived from R777. Western blot analysis showed that the Fab fragments specifically recognized LRP. Addition of up to 275 μg/ml of Fab showed no response (79 ± 9 nM vs 80 ± 13 nM, n=5 expts, 59 cells, NS, $p>0.05$). To further examine this issue, and to test the hypothesis that endocytosis is sufficient to evoke a calcium signal, neuronal responses to another LRP ligand, which is bound and readily endocytosed by LRP, but is monomeric, was examined. Lactoferrin is readily taken up by neurons via LRP (Qiu, Z., *et al., J. Neurochem. 73*:1393-8 (1999)), but lactoferrin (up to 5 μM) did not evoke a calcium response (79 ± 9 nM vs 89 ± 21 nM, n=4 expts, 32 cells, NS, $p>0.05$). These results demonstrate that although the Fab fragments and lactoferrin bound to the receptor, they were unable to activate calcium entry, thus supporting the hypothesis that dimerization of LRP may be important for the calcium signaling event.

It is possible that a2M* concentrations might provide information about the local microenvironment to neurons, and therefore might alter local dendritic calcium levels in a spatially restricted fashion. As a means of examining the spatial

characteristics of the response, several picoliters of a a2M* (5 μM) solution was applied via micropipette to various regions of individual neurons using a pressure pulse. The delivery of a2M* was restricted to a circular area with a radius of about 25 μm, and this was the only area that responded with a calcium increase. The response was localized, and decays within several tens of seconds. Indeed, a second pressure pulse was able to stimulate the same area again, without affecting the calcium concentration in the cell body. In a similar experiment, positioning the pipette near the cell body was capable of eliciting a calcium transient that was restricted to the cell body, and did not spread to the dendrites. Thus, the response can be spatially restricted, and may not lead to a global increase in calcium. Furthermore, the calcium response does not lead to a large cellular depolarization, which, if above threshold, would be expected to increase calcium everywhere in the cell.

Discussion

Members of the LDL receptor family, including LRP, have been studied extensively as multiligand endocytic receptors (Herz, J., *et al., EMBO J.* 7:4119-27 (1988); Herz, J., *J. Biol. Chem.* 265:21355-62 (1990); Bu, G., *et al., J. Biol. Chem.* 269:29874-82 (1994)). The data presented herein support several novel conclusions suggesting that LRP serves an unexpected role as a signaling receptor as well. Stimulation of neurons by the LRP ligand a2M* elicits a robust calcium response, which is local to the area of stimulation, and which is temporally linked to the stimulus. The response appears to require LRP receptor dimerization. The response is neuron specific, and is mediated through NMDAR channels. Non-neuronal cells containing LRP within the same culture wells did not respond to a2M* with calcium influx.

LRP is implicated as the mediator of this response because it is an a2M* receptor on neurons (Rebeck, G.W., *et al., Neuron* 11:575-80 (1993); Bu, G., *et al., J. Biol. Chem.* 269:29874-82 (1994)), and the effect is blocked by RAP

which is a specific physiologic inhibitor of the LDL family of receptors. That LRP is specifically involved is demonstrated by the observation that an antibody directed against the extracellular domain of LRP can also induce calcium influx in neurons. Of note, both a2M*, which is tetrameric, and the bivalent R777 antibody could potentially lead to dimerization of the receptor. The Fab fragments of the same antibody and a monomeric ligand, lactoferrin, did not evoke a calcium response, supporting the conclusion that dimerization may play a role in calcium signaling. LRP is present on both neurons and astrocytes in culture and in adult brain (Rebeck, G.W., *et al., Neuron 11*:575-80 (1993); Bu, G., *et al., J. Biol. Chem. 269*:29874-82 (1994); Bu, G., *et al., J. Biol. Chem. 269*:18521-8 (1994)), although only neurons have an LRP mediated calcium response. It is possible that there may be a neuron-specific intracellular adapter protein that mediates opening of NMDAR channels after LRP dimerization, or that LRP may interact directly with NMDA receptors.

Links between a2M* and calcium influx have been examined in several systems. Previous studies in macrophages (Misra, U.K., *J. Biol. Chem. 269*:18303-6 (1994)) and trabecular meshwork cells (Howard, G.C., *et al., Arch. Biochem. Biophys. 333*:19-26 (1996)) suggest 2 classes of a2M methylamine receptors: LRP and a separate "signaling receptor." Stimulation of the latter leads to a rapid rise in intracellular calcium in macrophages, which is not blocked by RAP or altered by LRP antibodies. By contrast, a2M* binding to LRP does not appear to induce a calcium influx in macrophages, consistent with the lack of response that was observed in non-neuronal cells.

In neurons, the role of LDL receptor family members in calcium influx may be complex. A rise in intracellular calcium in hippocampal neurons has also been observed after treatment with apolipoprotein E (Hartmann, H., *et al., Biochem. Biophys. Res. Commun. 200*:185-92 (1994)); these apolipoprotein E-evoked Ca^{2+} increases are dependent on extracellular calcium and blocked by the Ca^{2+}-channel antagonists nickel and ω-Agatoxin-IVa, implicating activation of P/Q type Ca^{2+}-channels (Muller, W., *et al., Brain Pathol. 8*:641-53 (1998)). More similar to the

current findings, proteolytic fragments of apolipoprotein E or a tandem dimer repeat peptide derived from apolipoprotein E, elicited calcium responses in both hippocampal cultures and chick sympathetic neurons, with the calcium increases being blocked by RAP and by the NMDA receptor antagonist MK-801 (Tolar, M., *et al., J. Neurosci. 19*:7100-10 (1999)). The relationship among these various experimental systems must be more closely examined, but the accumulating evidence strongly suggests that, in neurons, stimulation of LRP by ligands such as a2M* or apolipoprotein E leads to a calcium signaling event. It is possible that this acts as a neuronal sensor for proteolytic activity or lipid breakdown within a dendrite's microenvironment.

The influx of calcium into spatially segregated dendritic elements due to LRP-mediated activation of NMDAR channels is likely to locally impact a wide variety of downstream signaling cascades, including IP3, protein kinase C, and calcium/calmodulin dependent kinase (Alkon, D.L., *et al., Trends Neurosci. 21*:529-37 (1998)). This cascade implies that a2M* interaction with LRP may provide a novel mechanism of altering local dendritic excitability, and thus synaptic efficacy. a2M* has previously been implicated in inhibiting long term potentiation (Cavus, I., *et al., J. Neurosci. Res. 43*:282-8 (1996)). Another LRP ligand, tissue plasminogen activator (tPA), contributes to activity-dependent synaptic plasticity in the hippocampus via LRP (Zhuo, M., *et al., J. Neurosci. 20*:542-549 (2000)). It is possible that the LRP-mediated NMDAR channel activation and calcium influx that is seen might contribute to these phenomena.

In addition to its well established role as a multiligand endocytic receptor, there is some precedence for LRP having a role in neuronal signaling pathways. a2M*, apolipoprotein E, and other LRP ligands have been shown to promote neurite outgrowth via LRP (Holtzman, D.M., *et al., Proc. Natl. Acad. Sci. U.S.A. 92*:9480-4 (1995); Ishii, M., *et al., Brain Res. 737*:269-74 (1996); Mori, T., *et al., Brain Res. 567*:355-7 (1991); Postuma, R.B., *et al., FEBS Lett. 428*:13-6 (1998)). Moreover, the intracellular domain of LRP can bind both disabled and FE65, adapter proteins implicated in signal transduction (Trommsdorff, M., *et al., J.*

Biol. Chem. 273:33556-60 (1998)). LRP has also been reported to interact with a heterotrimeric G protein (Goretzki, L. and Mueller, B. M., *Biochem. J. 336*:381-6 (1998)). Treatment of LRP-expressing cell lines with the LRP ligands lactoferrin or urokinase-type plasminogen activator caused a significant elevation in cAMP and stimulated PKA activity in a dose-dependent manner (Goretzki, L. and Mueller, B. M., *Biochem. J. 336*:381-6 (1998)).

The idea that members of the LDL receptor family could act as signaling receptors (Cooper, J.A. & Howell, B.W., *Cell 97*:671-4 (1999)) in the central nervous system recently received dramatic support from the observation that APOER2/VLDL-r double null animals develop a reeler phenotype (Trommsdorff, M., *et al., Cell 97*:689-701 (1999)), due to inactivation of the reelin-disabled signaling pathway. Reelin is a ligand for APOER2 and VLDL-r, supporting the idea that these receptors directly mediate reelin signal transduction (Trommsdorff, M., *et al., Cell 97*:689-701 (1999); D'Arcangelo, G., *et al., Neuron 24*:471-9 (1999)). It is interesting to note that both VLDL-r and APOER2 are also strongly expressed on mature neurons (Christie, R.H., *et al., J. Neuropathol. Exp. Neurol. 55*:491-8 (1996); Clatworthy, A.E., *et al., Neurosci. 90*:903-11 (1999)), and APOER2 has been reported to be an a2M* receptor (Stockinger, W., *et al, J. Biol. Chem. 273*:32213-21 (1998)). The data regarding a specific anti-LRP antibody clearly implicate LRP itself, but do not rule out a role for APOER2 in calcium signaling. These data, taken together with the current demonstration that LRP is also a potent signaling receptor in neurons, suggest a major role for the LDL receptor family in brain development and function.

Several LRP ligands have been strongly implicated in the pathophysiology of Alzheimer's disease. Aβ, the major constituent of senile plaques, is a peptide fragment of the amyloid precursor protein, itself a protease inhibitor and ligand for LRP (Kounnas, M.Z., *et al., Cell 82*:331-40 (1995)). Apolipoprotein E and a2M* bind Aβ and the complexes can be cleared by LRP (Qiu, Z., *et al., J. Neurochem. 73*:1393-8 (1999); Jordan, J., *et al., J. Neurosci. 18*:195-204 (1998); Narita, M., *et al., J. Neurochem. 69*:1904-11 (1997)). Genetic studies strongly implicate

polymorphisms in the apolipoprotein E gene, and also in the a2M and the LRP genes, in affecting risk for late onset Alzheimer's disease (see Hyman, B.T., *et al., Arch. Neurol.* 57:646-650 (2000) for review). Finally, it should be noted that APP contains a NPXY domain in its carboxyl terminus, and APP can bind DAB1 and Fe65 adaptor proteins which also interact with LRP (Trommsdorff, M., *et al., J. Biol. Chem.* 273:33556-60 (1998)). The current observations that LRP may be both an endocytic and a signaling receptor may thus be of relevance to the role of LRP and its ligands in Alzheimer's disease.

What Is Claimed Is:

1. A method of treating a subject in need of treatment of a neurological disorder, said method comprising: administering to said subject a pharmaceutically effective amount of an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptors on neuronal cells, said binding modulating calcium influx in said neuronal cells.

2. The method of claim 1, wherein said calcium influx is mediated through an NMDA receptor on said neuronal cells.

3. The method of claim 1, wherein said agent that binds to LRP on said neuronal cells causes dimerization of LRP on said neuronal cells.

4. The method of claim 1, wherein said agent is selected from the group consisting of: protein-lipid complexes, proteases, protease inhibitors, protease/inhibitor complexes, intracellular proteins, LRP receptor antibodies and proteins that interact with LRP.

5. The method of claim 1, wherein said agent is selected from the group consisting of: activated alpha-2-macroglobulin, apolipoprotein E, and apolipoprotein E4.

6. The method of claim 5, wherein said agent is activated alpha-2-macroglobulin.

7. The method of claim 1, wherein said agent that binds to LRP on said neuronal cells does not reduce the amount or rate of binding of amyloid β-precursor protein (APP) with LRP.

8. The method of claim 1, wherein said neurological disorder is selected from the group consisting of: Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, amyotrophic lateral sclerosis, epilepsy, and stroke.

9. The method of claim 8, wherein said neurological disorder is Alzheimer's Disease.

10. A method of inhibiting cell death in neuronal cells, said method comprising: providing said neuronal cells with an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor on said neuronal cells, said binding modulating calcium influx in said neuronal cells.

11. The method of claim 10, wherein said calcium influx is mediated through an NMDA receptor on said neuronal cells.

12. The method of claim 10, wherein said agent is selected from the group consisting of: receptor associated protein (RAP), small peptides or molecules that bind LRP or mimic RAP, LRP receptor antibodies, and proteins that interact with LRP.

13. A method of modulating calcium influx in neuronal cells, said method comprising: providing said neuronal cells with an agent that binds to low-density lipoprotein receptor-related protein (LRP) receptor on said neuronal cells, said binding modulating calcium influx in said neuronal cells.

14. The method of claim 13, wherein said calcium influx is mediated through an NMDA receptor.

15. The method of claim 13, wherein said agent is selected from the group consisting of: protein-lipid complexes, proteases, protease inhibitors,

protease/inhibitor complexes, intracellular proteins, LRP receptor antibodies, and proteins that interact with LRP.

16. The method of claim 13, wherein said agent is an agonist of said LRP receptor.

17. The method of claim 16, wherein said agonist is activated alpha-2-macroglobulin.

18. The method of claim 16, wherein said agonist is an antibody that binds said LRP receptor.

19. The method of claim 18, wherein said antibody is R777.

20. The method of claim 13, wherein said agent is an antagonist of said LRP receptor.

21. The method of claim 20, wherein said antagonist is selected from the group consisting of: receptor associated protein (RAP), small peptides or molecules that mimic RAP, LRP antibodies, and proteins that interact with LRP.

22. A method of identifying an agent that modulates calcium influx in neuronal cells by binding to low-density lipoprotein receptor-related protein (LRP) receptor on said neuronal cells, said method comprising:

a) treating neuronal cells with an agent and assaying for calcium influx;

b) treating neuronal cells with a known modulator of LRP-mediated calcium influx and said agent and assaying for calcium influx; and

c) comparing the levels of calcium influx in (a) and (b) to determine if said agent modulates calcium influx by interacting with an LRP receptor.

23. The method of claim 22, wherein said calcium influx is mediated through an NMDA receptor on said neuronal cells.

24. The method of claim 22, wherein (b) is performed on said neuronal cells of (a) after said agent in (a) is removed.

25. The method of claim 23, wherein said known modulator of LRP-mediated calcium influx is an inhibitor of said LRP-mediated calcium influx.

26. The method of claim 23, wherein said known modulator of LRP-mediated calcium influx is a stimulator of said LRP-mediated calcium influx.

27. The method of claim 25, wherein said known modulator of LRP-mediated calcium influx is an NMDA receptor channel antagonist.

28. The method of claim 27, wherein said NMDA receptor channel antagonist is selected from the group consisting of: MK-801, D(-)-2- Amino- 5-phosphonopentanoic acid, D(-)- 2- Amino- 4- phosphonobutyric acid, ketamine, ifenprodil and phencyclidine.

29. The method of claim 28, wherein said NMDA receptor channel antagonist is MK-801.

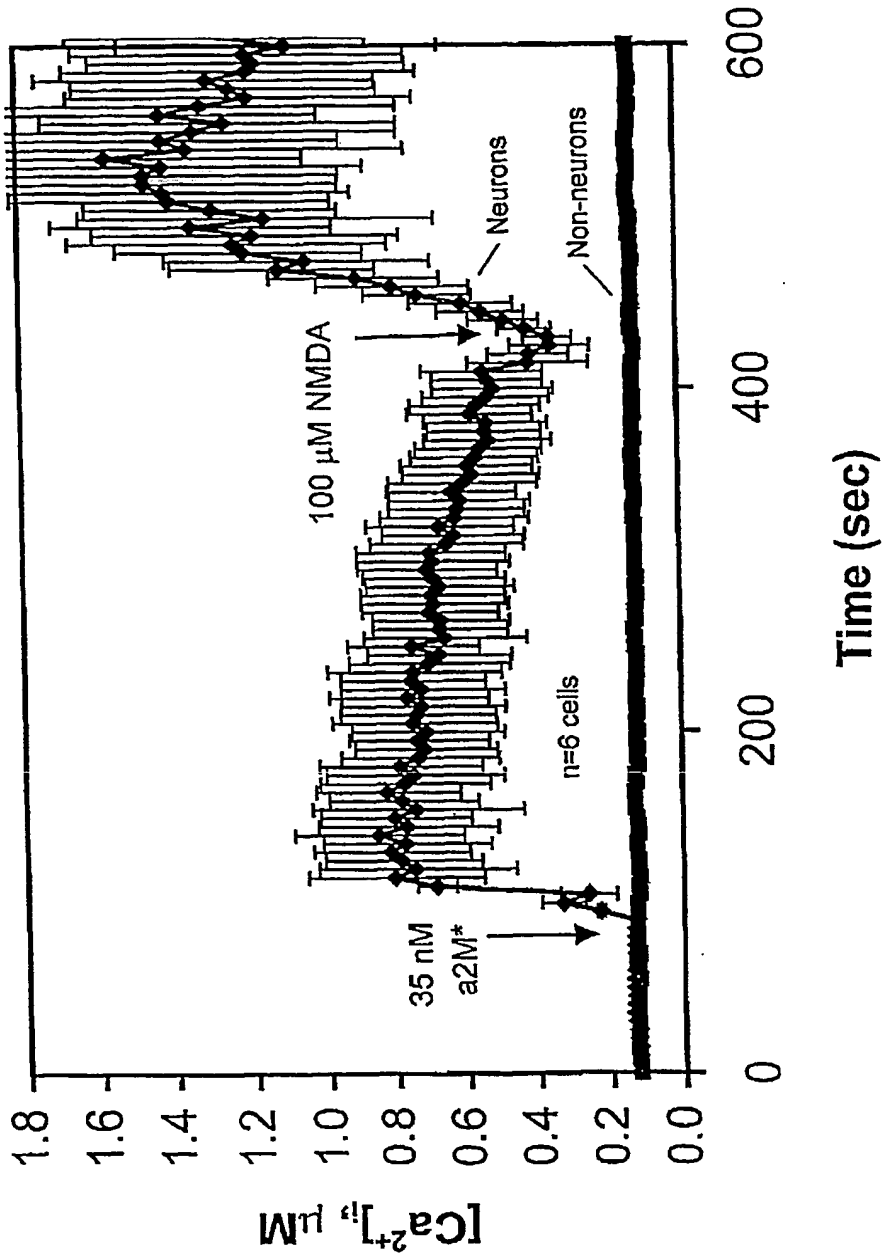
[Ca2+]i, µM
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
Time (sec)
100 µM NMDA
35 nM a2M*
Neurons
Non-neurons
n=6 cells

FIG. 1

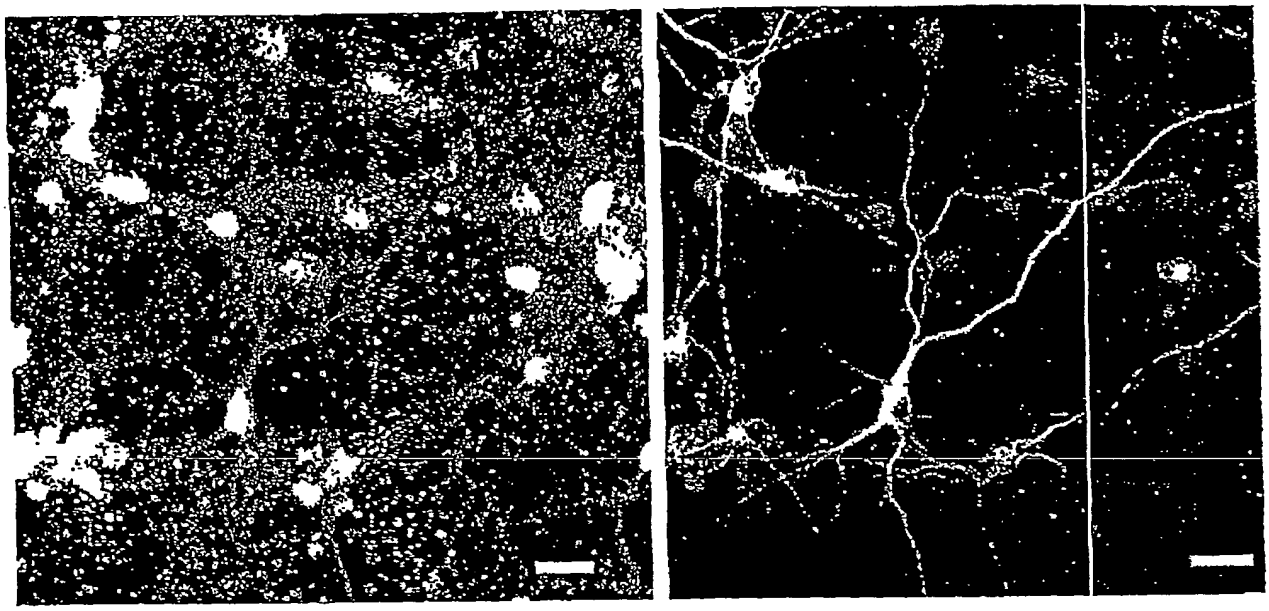

FIG. 2

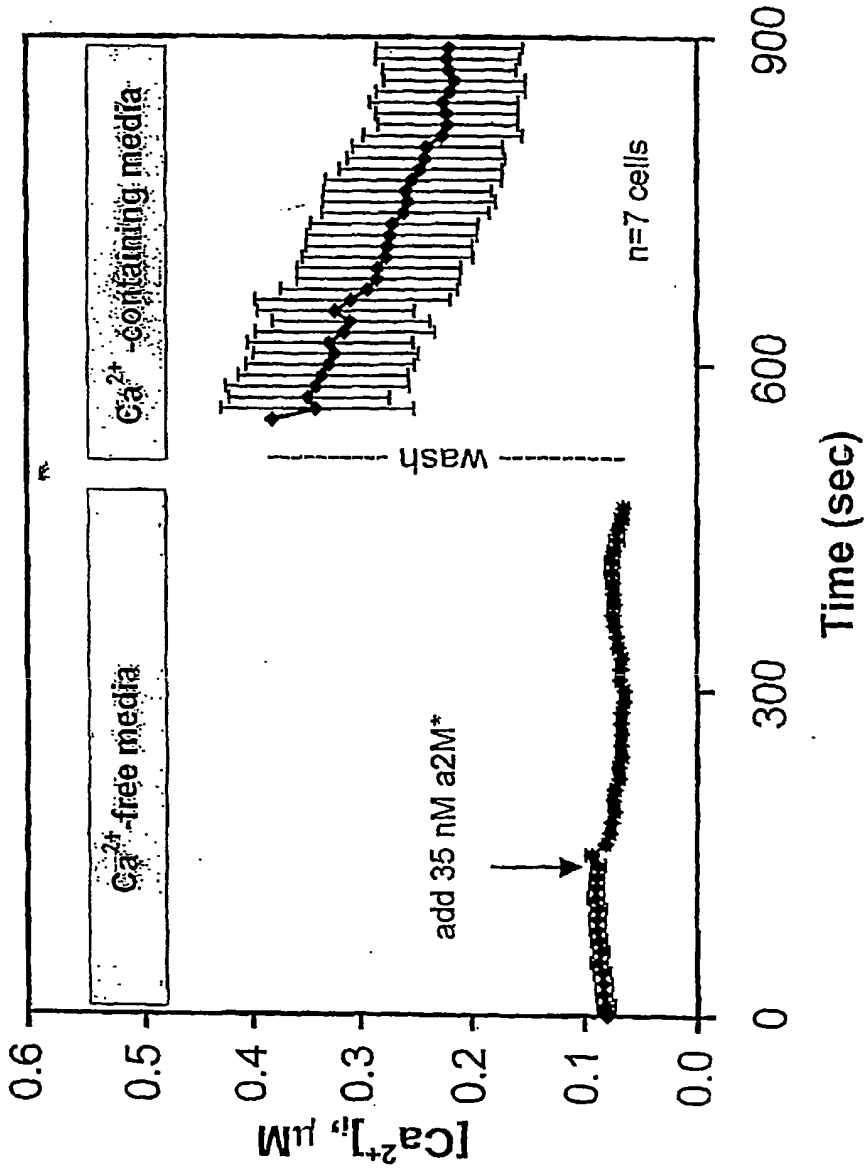
$[Ca^{2+}]_i$, μM
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
300
600
900
Time (sec)
Ca^{2+}-free media
Ca^{2+}-containing media
wash
add 35 nM a2M*
n=7 cells

FIG. 3

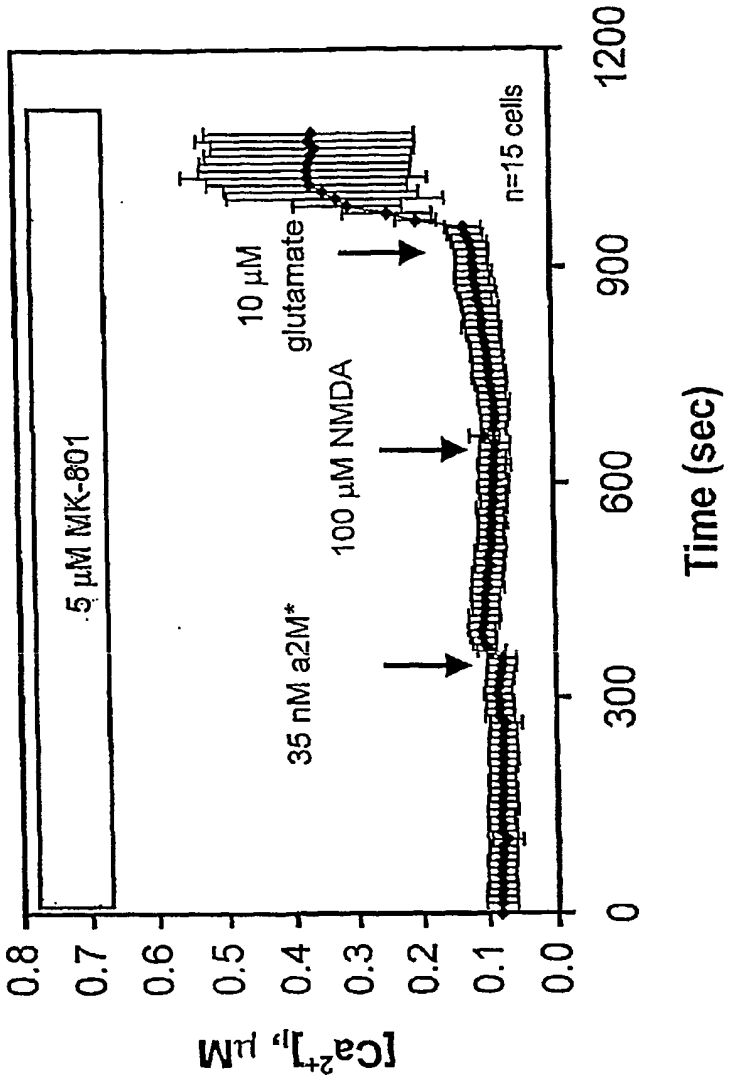
[Ca2+]i, µM
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
300
600
900
1200
Time (sec)
.5 µM MK-801
35 nM a2M*
100 µM NMDA
10 µM glutamate
n=15 cells

FIG. 4

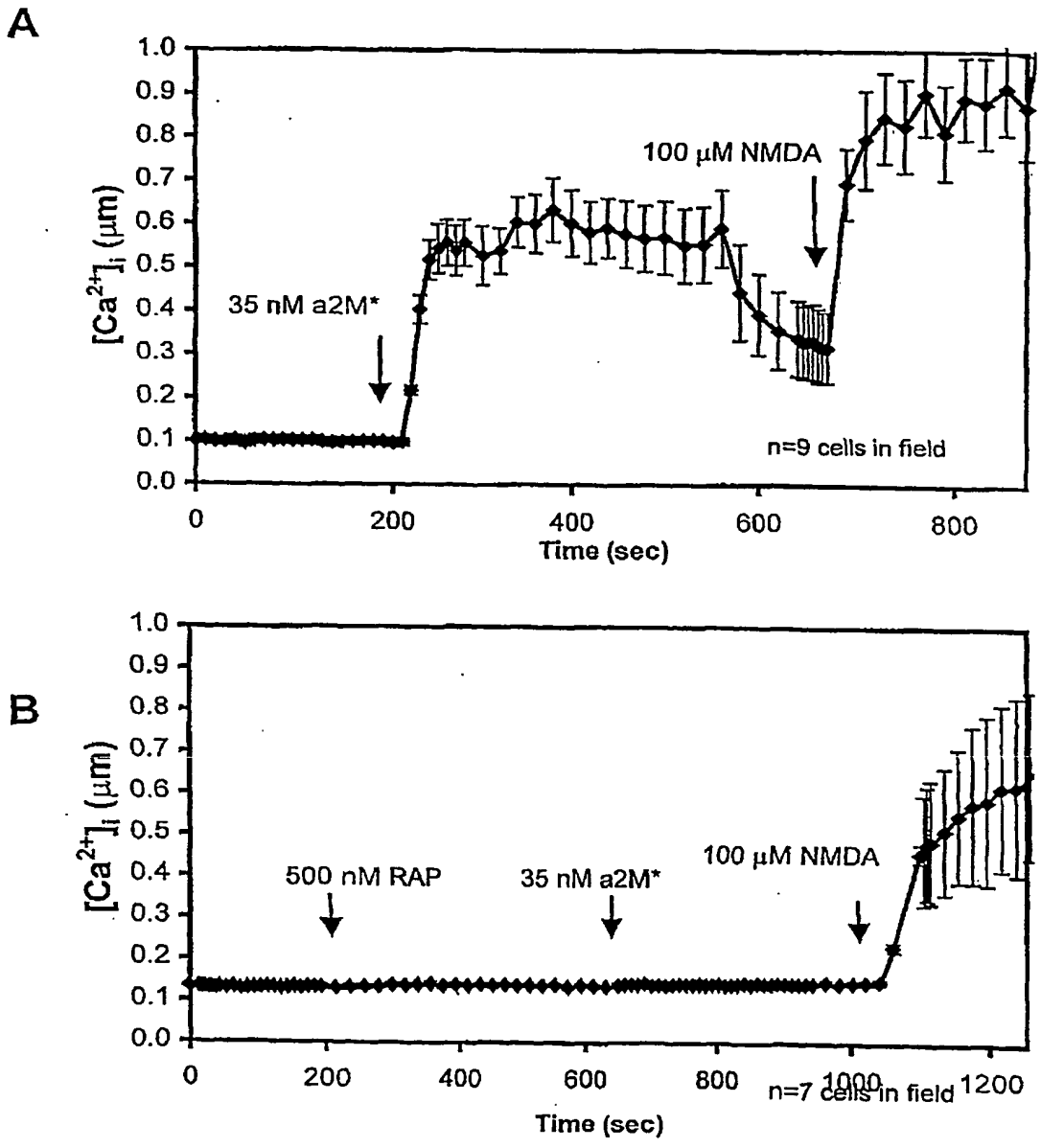
A
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[Ca2+]i (μm)
35 nM a2M*
100 μM NMDA
n=9 cells in field
0
200
400
600
800
Time (sec)
B
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[Ca2+]i (μm)
500 nM RAP
35 nM a2M*
100 μM NMDA
n=7 cells in field
0
200
400
600
800
1000
1200
Time (sec)

FIG. 5

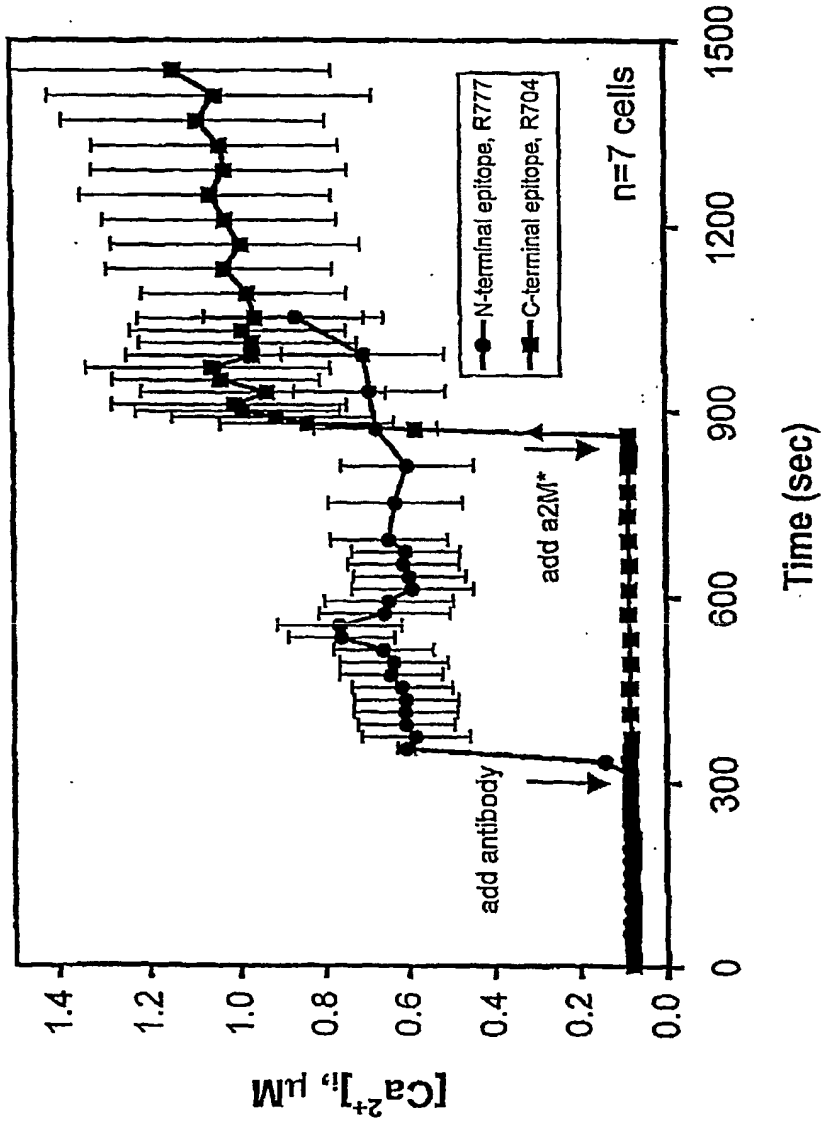
[Ca2+]i, μM
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
300
600
900
1200
1500
Time (sec)
n=7 cells
N-terminal epitope, R777
C-terminal epitope, R704
add antibody
add a2M*

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/40636

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17 A61K38/55 A61K38/57 G01N33/92 A61K39/395
A61K38/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 46246 A (GEN HOSPITAL CORP ;SAUNDERS ALEISTER J (US); KOVACS DORA M (US); T) 10 August 2000 (2000-08-10) claims 1-3,27,29,30,33,66	1-17
X	BUTTINI M ET AL.: "Expression of human apolipoprotein E3 or E4 in the brains of Apoe-/- mice: isoform: specific effects on neurodegeneration" THE JOURNAL OF NEUROSCIENCE, vol. 19, no. 12, 1999, pages 4867-4880, XP002178437 page 4867, left-hand column, paragraph 1 - paragraph 2	10-12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2001	03/12/2001

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/40636

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TOLAR M ET AL.: "Truncated apolipoprotein E (ApoE) causes increased intracellular calcium and may mediate ApoE neurotoxicity" THE JOURNAL OF NEUROSCIENCE, vol. 19, no. 16, 15 August 1999 (1999-08-15), pages 7100-7110, XP002178636 cited in the application the whole document	13-16, 20-23, 25,27-29
X	BACKSKAI BJ ET AL.: "Alpha-2-macroglobulin (a2M*) activation of low density lipoprotein receptor related protein (LRP) increases intracellular calcium in cortical neurons" SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 25, no. 1-2, - 1999 page 1347 XP001024466 the whole document	22-25, 27-29
X	XIAO-SHU WANG ET AL.: "Rapid elevation of neuronal cytoplasmic calcium by apolipoprotein E peptide" JOURNAL OF CELLULAR PHYSIOLOGY, vol. 173, 1997, pages 73-83, XP001024477 page 73, right-hand column -page 74, left-hand column, paragraph 2 page 78, right-hand column; figure 9 page 76, left-hand column, last paragraph figure 9 page 82, left-hand column, line 4 - line 7	13,15, 16, 20-22, 24,25
A	MAHMOOD HUSSAIN M ET AL. : "The mammalian low-density lipoprotein receptor family" ANNUAL REVIEWS IN NUTRITION, vol. 19, 1999, pages 141-172, XP001024478 abstract table 2 page 157, last paragraph -page 158 table 1 page 148, last paragraph -page 151, paragraph 1	1-29
A	POSTUMA RB ET AL.: "Effects of the amyloid protein precursor of Alzheimer's disease and other ligands of the LDL receptor-related protein on neurite outgrowth from sympathetic neurons in culture" FEBS LETTERS, vol. 428, 1998, pages 13-16, XP001024471 abstract page 13 page 15, left-hand column	1-9

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/40636

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PEDERSEN WA ET AL.: "A mechanism for the neuroprotective effect of apolipoprotein E: isoform-specific modification by the lipid peroxidation product 4-hydroxynonenal" JOURNAL OF NEUROCHEMISTRY, vol. 74, 2000, pages 1426-1433, XP002178438 abstract page 1426, left-hand column, paragraph 1 ---	1-9
A	LOVESTONE S: "Early diagnosis and the clinical genetics of alzheimer's disease" JOURNAL OF NEUROLOGY, vol. 246, no. 2, 1999, pages 69-72, XP001024584 abstract ---	1-9
A	TROMMSDORFF M ET AL.: "Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 50, 1998, pages 33556-33560, XP002178637 cited in the application the whole document -----	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-4, 7, 10, 11 and 13- 15 relate to methods using compounds defined by reference to a desirable characteristic or property, namely:
- binding to LRP on neuronal cells, said binding modulating calcium influx in said cells

The claims cover all methods using compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds: alpha-2-macroglobulin, apolipoprotein E and E4, LRP-specific antibodies, RAP.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/40636

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0046246 A	10-08-2000	AU 2865000 A	25-08-2000
		WO 0046246 A1	10-08-2000

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